LESSON 7:
SINGLE CELL CULTURES

Objective

The objective of this Lesson is to isolate single cells or free cells and culture them, using various techniques as described in the coming pages. Single cell cultures are excellent systems to study the properties and potentialities of plant cells. Such types of cultures are perfect materials for obtaining secondary metabolites in cultures.

Introduction

Establishment of single cell cultures provides an excellent opportunity to investigate the properties and potentialities of plant cells. Such systems contribute to our understanding of the interrelationships and complementary influences of cells in multicellular organisms. The pioneering attempts made by Haberlandt (see Lesson1) failed to achieve divisions in free cells but his detailed paper in 1902 stimulated further studies in this area. Subsequently, several workers reported spectacular success in achieving isolated single cell division and even raised complete plants from single cell cultures. This generated much interest among plant biotechnologists who recognised the merits of applying cell cultures over an intact organ or whole plant cultures to synthesise natural products. Using cell cultures in studies designed to describe the pathways of cellular metabolism was another aspect that initially attracted the attention of plant biologists. It was soon realised that single cell systems have a great potential for crop improvement. Free cells in cultures permit quick administration and withdrawal of diverse chemicals/substances, thereby making them easy targets for mutant selection. Moreover, the individual cells within a population of cultured cells invariably show cytogenetical and metabolic variations depending on the stage of the growth cycle and culture conditions. Such variability, termed 'spatial heterogeneity' (Lindsey and Yeoman 1985), has been the subject of much interest since differences between cells in their karyotype and the ability to accumulate secondary metabolites would manifest during morphogenesis in the clones regenerated from single cells. In this way the cell line selection technique can be usefully applied to produce high-yielding cultures as well as plants with superior agronomic traits.

Isolation Of Single Cells

From Plant Organs

The most suitable material for the isolation of single cells is the leaf tissue since a more or less homogeneous population of cells in the leaves offer good candidates for raising defined and controlled large-scale cell cultures. From such intact plant organs (as leaf tissue) single cells can be isolated using mechanical or enzymatic methods.

I. Mechanical Method

Gnanam and Kulandaivelu (1969) developed a procedure which has since been successfully used to isolate mesophyll cells, active in photosynthesis and respiration, from mature leaves of several species of dicots and monocots including the grasses. Even metabolically active single cells from the bundle sheath of crab grass (Digitaria sanguinalis) can be isolated using a similar procedure. The procedure involves mild maceration of 10 g leaves in 40 ml of the grinding medium (20 ul mol sucrose, 10u mol $MgCI_{2,}$ 20u mol tris-HCI buffer, pH 7.8) with a mortar and pestle. The homogenate is passed through two layers of muslin cloth and the cells thus released are washed by centrifugation at low speed using the same medium.

The mechanical isolation of free parenchymatous cells can also be achieved on a large scale by this method.

Ii. Enzymatic Method

In 1968, Takebe and his co-workers treated tobacco leaf tissue with the enzyme pectinase and obtained a large number of metabolically active cells. A point to note is that potassium dextran sulphate in the enzyme mixture improved the yield of free cells.

Isolation of single cells by the enzymatic method has been found convenient as it is possible to obtain high yields from preparations of spongy parenchyma with minimum damage or injury to the cells. This can be accomplished by providing osmotic protection to the cells while the enzyme macerozyme degrades the middle lamella and cell wall of the parenchymatous tissue. Applying the enzymatic method to cereals (Hordeum vulgare, Zea mays) has proven rather difficult since the mesophyll cells of these plants are apparently elongated with a number of interlocking constrictions, thereby preventing their isolation.

From Cultured Tissues

The most widely applied approach is to obtain a single cell system from cultured tissues. Freshly cut pieces from surface-sterilised plant organs are simply placed on a nutrient medium (solidified) consisting of a suitable proportion of auxins and cytokinins to initiate cultures. Explants on such a medium exhibit callusing at the cut ends, which gradually extends to the entire surface of the tissue. The callus is separated from an explant and transferred to a fresh medium of the same composition to enable it to build up a mass of tissue. Repeated subculture on an agar medium improves the friability of the callus, a prerequisite for raising a fine cell suspension in a liquid medium. The pieces of undifferentiated and friable callus are transferred in a continuously agitated liquid medium dispensed in autoclaved flasks or other suitable vials. Agitation is done by placing the culture flasks/vials on an orbital-platform shaker or suitable device. Movement of the culture medium exerts mild pressure on small pieces of tissue, breaking them into free cells and small cell aggregates. Further, it augments the gaseous exchange between the culture medium and the culture air, and also ensures uniform distribution of cells as well as cell clumps in the medium.

Single cells can also be isolated from suspension cultures by suitably filtering out cell clumps and harvesting the cells by centrifugation. A fine suspension of cells is usually obtained particularly when friable calli are used for the initiation of suspension cultures.

Cloning

A clone of cells consists of all the cells derived through mitosis from a single cell and the process of obtaining a clone is called cloning. Therefore, all the cells of clone are expected be identical with each other in their genotype and karyotype (chromosome constitution), and other attributes, except for the changes that may arise afresh during and after cloning. Cloning is based on single cells separated from tissues and cultured in a manner to allow separate recovery of the cell mass derived from them.

Culture of Single Cells

Single cells can be cultured using the following techniques:

1. filter paper raft nurse tissue technique,
2. microchamber technique,
3. microdrop method,
4. Bergman's plating technique and
5. thin layer liquid medium.

Isolated single cells fail to divide in normal tissue culture media. Therefore, either a nurse tissue or a conditioned medium is used for their culture. A conditioned medium is a medium in which plant cells have been grown for about 48 hr (cells are then filtered out).

Let us understand each technique in detail as follows:

Filter Paper Raft-Nurse Tissue Technique:

Single cells are placed on small pieces (8 x 8 mm) of filter paper (sterilized) which themselves are placed on top of established callus cultures several days in advance. This allows the filter papers to be wetted by the exudates from callus tissues. The single cells placed on the filter papers (Fig.1) derive their nutrition from the callus exudates diffusing through the filters. The cells divide and form macroscopic colonies on the filters; the colonies are isolated and cultured.

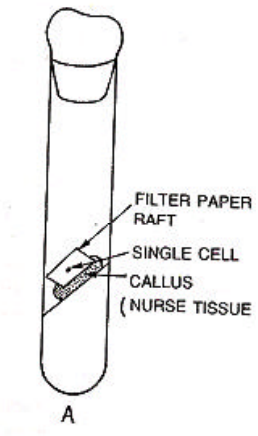

Fig. 1. Filter paper raft- nurse Technique

Microchamber Technique

A microchamber can be created either by using a microscope slide and coverslips (latter held in place by sterile mineral oil), or by a cavity slide (Fig.2). Single cells are suspended in conditioned medium, and a drop of medium having a single cell is placed in the microchamber which is covered by a coverslip. In case of cavity slide, the drop is placed onto a coverslip which is then inverted into the slide cavity. Microchambers allow microscopic observation, and they can be kept in a petricdish for incubation.

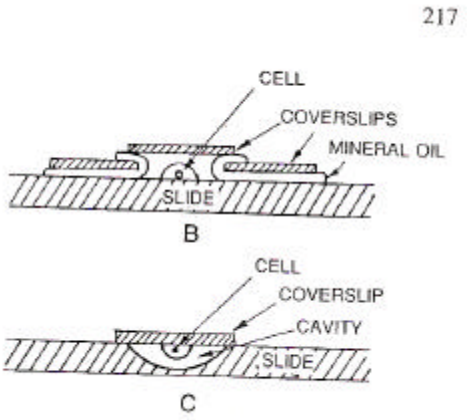

Fig. 2. Microchamber Techniques

Microdrop Method

A specially designed dish, Cuprak dish, having a smaller outer chamber (to be filled with sterile distilled water to avoid desiccation of cells) and a larger chamber (having several numbered microwells) is employed. Microdrops of 0.25-0.5 ml are distributed in the microwells and the dish is sealed with parafilm (Fig. 3). Cell density in the medium is so adjusted as to give, on an average, one cell per droplet (it works out as 2-4 x 10^3 cells/ml). This method has been successfully used for protoplasts and should work with single cells as well.

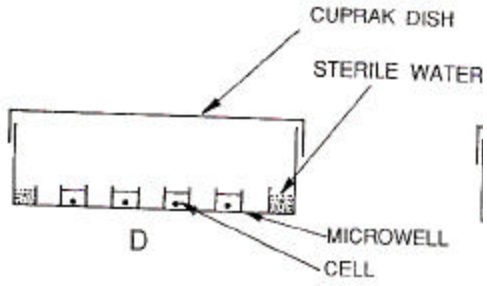

Fig. 3. Microdrop Technique

Bergmann's Plating Technique

In this widely used technique, cells are suspended in a liquid medium at a cell density that is twice the desired density in the plate. Sterilized agar (1 %) medium is kept melted in a water bath at 35°C. Equal volumes of the liquid and agar media are mixed thoroughly and quickly spread in 1mm thick layer in a

Petri dish. The cells remain embedded in the soft agar medium, are observable under a microscope, and when macroscopic colonies develop they are isolated and cultured separately.

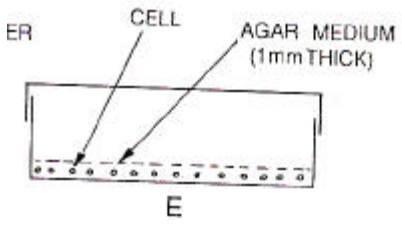

Fig. 4. Bergmann's Plating Technique

Thin Layer Liquid Medium Culture

Cells can be plated in a thin layer of liquid medium to allow adequate aeration. Since cells are not fixed in position, it is not possible to follow up an individual cells during culture. This technique is common for protoplast culture.

What are the Culture Requirements?

It is important to:

a. culture the single cells in dark and keep microscopic observations to the minimum since light has a detrimental effect on cell proliferation. In addition,

b. either a conditioned medium or a suitably enriched medium should be used since standard tissue culture media are unsuitable. Some highly enriched synthetic media permit the culture of as few as 25-50 cells/ml, while some media supplemented with casamino acids and coconut milk support cell division at 1-2 cells/ml. It is postulated that some biochemicals essential for cell division leach into the culture medium when they are subcultured; this produces the lag phase (already described in previous Lesson). Cells begin to divide only when an equilibrium is established for these metabolites between the medium and the cells; this happens much later at lower cell densities and below a critical cell density it may not be achieved. For this reason conditioned or specially enriched media are required for culture of cells at low densities.

Cell Viability Test

Cell viability can be determined by anyone of the following approaches:

1. phase contrast microscopy,
2. staining with 2, 3, 5-triphenyltetrazolium chloride (TTC), or
3. fluorescein diacetate (FDA), or
4. Evan's blue.

Live cells show cytoplasmic streaming and a well defined healthy nucleus which are easily observable with a phase contrast microscope or even a light microscope. Cell masses can be stained with 1-2% solution of TTC which is reduced by living cells to formazan which yields red colour. Formazan can be extracted and measured with a spectrophotometer to give a quantitative estimate of viability, but it is not suitable for single or few cells.

Cell are treated with 0.01 % solution of FDA. Live cells cleave FDA by esterase activity and produce fluorescein, which cannot cross plasma membrane. With UV exposure, fluorescein gives green fluorescence so that live cells appear green, while dead cells do not fluoresce. Evan's blue (0.025%) is not taken up by live cells, while it freely enters into damaged dead cells. Therefore, all cells that take up stain are dead. Evan's blue is usually used in conjunction with FDA.

Conclusion

The technique of cloning single cells in vitro has demonstrated the fact that somatic cells, under appropriate conditions, can differentiate to a whole plant. A single cell has the potential to grow and develop a multicellular or multiorganed higher organism (totipotency of a cell). Since the potential lies mainly in cellular differentiation, this indicates that all genes responsible for differentiation are present within individual cells and many of them that remain inactive in differentiated tissues or organs are able to express only under adequate culture conditions.

Questions

1. Explain why single cells are important in cultures.
2. How will you isolate single cells for culture?
3. Describe the various techniques used for culture of single cells.

Note